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In Vitro Augmentation of Antitumor Effect in Combination with CPT-11 and CDDP for Human Colorectal Cancer

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Background and Objectives: Irinotecan hydrochloride (CPT-11) is one of the camptothecin analogues that has shown a broad spectrum of strong antitumor effectiveness against various cancers, including colorectal cancer. In order to promote the clinical response of chemotherapy for colorectal cancer using CPT-11, one of the most effective strategies is to use it in combination with other anticancer agents. In the present study, anticancer effects after combining CPT-11 and other antitumor agents were determined by a 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay of colorectal cancer cells, especially freshly isolated cancer cells.

Methods: Freshly isolated cancer cells from 20 patients with colorectal cancer and the established colon cancer cell lines were used in this study. The augmentation of the antitumor effectiveness of 7-ethyl-10-hydroxy-CPT (SN-38) was analyzed in combination with other anticancer agents. Furthermore, the antitumor effectiveness using lower concentrations of anticancer agents was measured to understand the mechanism of the augmentation.

Results: The percent inhibition of SN-38 in combination with cisplatin (CDDP) and mitomycin revealed a high anticancer effect compared with each anticancer agent alone for freshly isolated rectal cancer. CDDP also had a synergistic effect in combination with SN-38 according to the fractional product concept. At lower than plasma peak concentrations of SN-38, the anticancer effects were augmented in combination with lower concentrations of CDDP for freshly isolated colorectal cancer. This augmentation showed a strong synergistic effect.

Conclusions: These results may be supportive to ongoing clinical studies of chemotherapy by using CPT-11 and CDDP for advanced colorectal cancer.

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KEY WORDS: CPT-11; colorectal cancer; MTT assay; CDDP; MMC; SN-38

INTRODUCTION

One type of standard chemotherapy for colorectal cancer in the United States and Europe is fluorouracil (FU) or a FU combination therapy, whereas in Japan biomodulation of FU using cisplatin (CDDP) is the core of the chemotherapy treatment for colorectal cancer. On the other hand, one of the semisynthetic analogues of camptothecin, 7-ethyl-10-[4-(1-piperidyl)-1-piperidyl] carbonyloxy-camptothecin (irinotecan hydrochloride, CPT-11), was reported to be clinically effective as a second

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line of chemotherapy for colorectal cancer, especially for FU–resistant cases [1]. CPT-11 had a broad spectrum of strong antitumor effectiveness for colorectal cancer [2], leukemia, lymphoma [3], small cell and non–small cell lung cancer [4,5], and ovarian and cervical cancers [6] with less unpredictable side effects than the parent compound [7,8]. Metabolic activation of CPT-11 by a carboxylesterase was essential for its antitumor effectiveness. One of the active metabolites was identified as 7-ethyl-10-hydroxy-CPT (SN-38) [9]. SN-38 showed a 10–100 times stronger antitumor effect in vivo, compared to that of CPT-11. The anticancer effect of CPT-11 revealed that a single strand of DNA was inhibited in rebinding by making the stable complex of SN-38 + topoisomerase I, an enzyme that is related to DNA-DNA binding [10].

The augmentation of the antitumor effect of CPT-11 in combination with other anticancer agents was one way to promote its clinical efficacy. For advanced colorectal cancer, CDDP was one of the more potent candidates used in combination with CPT-11 in order to augment any anticancer effect. Regarding in vitro studies, SN-38 combined with CDDP showed synergistic antitumor effects for small cell and non–small cell lung cancer [11,12]. Furthermore, clinical phase II studies of CPT-11 and CDDP have been performed against advanced colorectal cancer, and their synergistic antitumor effectiveness was shown in the established colon cancer cell lines [13]. For ovarian cancer, clinical responses in combination with CPT-11 and CDDP were also reported to be higher than CPT-11 alone [14]. The antitumor effect of CPT-11 combined with other anticancer agents was analyzed in vitro for various kinds of cancer [15–17]. However, to our knowledge, most reports used the established tumor cell lines. When clinical applications are considered, freshly isolated human cancer cells should be used for the estimation. In the present study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, a rapid and quantitative colorimetric system for determining chemosensitivity in vitro [18,19], was utilized to measure the anticancer effect. Furthermore, the augmentation of anticancer effect of SN-38 in freshly isolated human colorectal cancer was analyzed in combination with other anticancer agents.

MATERIALS AND METHODS

Patients

Twenty patients (10 males and 10 females) with colorectal cancer were enrolled in this study. There were 7 patients with colonic cancer and 13 patients with rectal cancer, and the average age was 63.2 ± 8.7 years. Surgical specimens were obtained from 17 patients with primary colorectal lesions, 1 patient with a tumor of local recurrence, 1 patient with pleuritis carcinomatosa, and 1 patient with a metastatic liver tumor.

TABLE I. Tumor Characteristics Used in This Study

Characteristics	No. of patients
Primary cancer	
Colon	7
Rectum	13
Well differentiated adenocarcinoma	11
Histology	
Moderately differentiated adenocarcinoma	5
Poorly differentiated adenocarcinoma	1
UICC stage	
I	2
II	7
III	4
IV	7
Depth	
≦ss(a1)	12
>se(a2)	5
Lymph node metastasis	
0	11
≧1	6
Ly factor	
0	2
≧1	15
V factor	
0	3
≧1	14

Pathological examinations of primary colorectal tumors revealed that 11 had well-differentiated adenocarcinomas, 5 had moderately differentiated adenocarcinomas, and 1 had a poorly differentiated adenocarcinoma. The clinical stages according to the classification of malignant tumors by UICC in colorectal cancer were: 2 in stage I, 7 in stage II, 5 in stage III, and 7 in stage IV (Table I).

Tumor specimens were taken for diagnosis or therapeutic indications. Informed consent was obtained from patients for the use of samples for drug sensitivity testing, in accordance with the guidelines of the Ethical Committee on Human Research, Wakayama Medical School (Wakayama, Japan).

Tumor Cell Lines

The established colonic cancer cell lines used were LoVo and WiDr. These colonic cancer cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Chemicals

The chemicals tested were CPT-11, kindly provided by Daiichi Pharmaceutical Co. (Tokyo); SN-38, an active form of CPT-11 in vivo, provided by Daiichi; CDDP, provided by Nippon Kayaku (Tokyo); mitomycin, provided by Kyowa Hakko Tokyo (Tokyo); and doxorubicin and FU, provided by Kyowa Hakko Tokyo. The complete medium used consisted of RPMI-1640 (Nissui Co., Tokyo) supplemented with 10% heat-inactivated fetal

calf serum (GIBCO, Grand Island, NY), 2 m*M* L-glutamine, and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin).

Purification of Fresh Human Colorectal Cancer Cells

Freshly excised tumor tissues were processed using enzymatic digestion, as previously described [20,21]. Briefly, the tumor tissues were dissected into pieces <2 mm^3, which were immersed in a complete medium containing collagenase (2 mg/ml, type V-S; Sigma, St. Louis, MO), hyaluronidase (10 U/ml type IV-S; Sigma), and DNase-I (0.4 mg/ml; Sigma). After 40 min of incubation at 37°C, the cells were harvested, washed, and suspended in the complete medium. The purification of autologous tumor cells has also been previously described [20–22]. Tumor cells obtained from solid tumor specimens were centrifuged on ficoll-hypaque (specific gravity, 1.077; Pharmacia, Uppsala, Sweden) gradients at 400 *g* for 30 min in 50-ml tubes. The interface was collected and suspended at a concentration of 1×10^6/ml in a complete medium.

The cells were then layered on discontinuous gradients consisting of 10 ml of 100% and 15 ml of 75% ficoll-hypaque in 50-ml tubes. After centrifugation at 400 *g* for 30 min, a tumor cell–rich fraction was collected from the 75% interface. The tumor cell–enriched suspension was then layered onto discontinuous gradients containing 4 ml each of 25%, 15%, and 10% Percoll (Pharmacia) in a complete medium in 15-ml plastic tubes. Centrifugation was performed at 25 *g* for 7 min, and tumor cells depleted of lymphoid cells were collected from the bottom and from the 25% interface. Then they were washed and resuspended in the complete medium at a concentration of 1×10^6/ml. The cells were at least 90–95% viable as determined by the trypan blue dye exclusion test.

Method of MTT Assay

Antitumor effects were assessed using tetrazolium salt MTT (Sigma, no. M2128) to measure the viability of the tumor cells [20–22]. The tumor cells were at least 90–95% viable as determined by the trypan blue dye exclusion test [21]. One hundred microliters of suspended tumor cells (fresh human cancer cells, 1×10^6 cells/ml; and an established human cancer cell line, 1×10^5 cells/ml) was added to 25 μl of each drug at the specified concentration described further in Results in 96-well Corning microtiter plates (no. 25860; Corning, NY). In order to equalize the tumor and drug concentration, 25 μl of the complete medium was added with one-drug exposure, and no complete medium was added in the two-drug combination. Then these plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 96 h. The chemosensitivity assay was assessed in triplicate. Three microtiter wells containing tumor cells suspended in 150 μl of the complete medium (the total number of tumor cells was equivalent to that in the test wells) were used as controls for cell viability, and three wells containing only complete medium were used as controls for nonspecific dye reduction. After incubation, the plates were centrifuged, the supernatants were removed, and 30 μl/well MTT solution with 10 μ*M* sodium succinate was added to all the wells. The plates were incubated for an additional 4 h, and 150 μl of dimethyl sulfoxide (DMSO) was then added to all the wells [23]; the mixtures were pipetted thoroughly to dissolve the dark blue crystals.

The plates were then read on a microplate reader (MTP-32, Corona Electric, Ibaragi, Japan) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The control wells without tumor cells had an OD of <0.005, and the samples in which the OD was >0.1 were accepted for the assay. The inhibition rate was calculated as follows: inhibition rate = [1 − (OD drug treated/OD control)] × 100.

Analysis of Combination's Effect

The effect of the drug combination was determined by referring to the fractional product (fp) concept as follows [24,25]: fp value = I1 + 2/(I1 + I2 − I1 × I2) >1 = synergistic, 1 = additive, and <1 = antagonistic. I1 and I2 were the mean inhibition rates of each single drug, I1 + 2 is the mean inhibition rate obtained by a two-drug combination.

Statistical Analysis

Significant differences were determined by a nonparametric Wilcoxon signed-rank test and a Mann-Whitney test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Augmentation of Anticancer Effects in Combination with SN-38

Anticancer effects in combination with SN-38 and other conventional anticancer agents (CDDP, FU, mitomycin, and doxorubicin) were analyzed for freshly isolated colorectal cancer cells using an MTT assay. The percent of inhibition rates, as they relate to the anticancer effects, of SN-38 alone, CDDP alone, FU alone, mitomycin alone, and doxorubicin alone, and that of the combination with SN-38 for colonic cancer and rectal cancer are indicated in Table II. For rectal cancer, the percent of inhibition rates of SN-38 alone, CDDP alone, and SN-38 plus CDDP were 53.8 ± 37.4, 74.2 ± 14.9, and 85.9 ± 8.8, respectively ($P < 0.01$; SN-38 alone, CDDP alone vs. SN-38 plus CDDP). Furthermore, the percent of inhibition rates of SN-38 alone, mitomycin alone, and SN-38 plus mitomycin were 53.8 ± 37.4, 78.5 ± 13.2, and 86.7 ± 11.5, respectively ($P < 0.01$; SN-38 alone, mitomycin alone, vs. SN-38 plus mitomycin). For colon cancer,

TABLE II. Percent Inhibition of SN-38 Combined With Other Anticancer Agents in Freshly Isolated Coloretal Cancer Cells*

		% Inhibition				
	Combination with SN-38	SN-38	CDDP	FU	Mitomycin	Doxorubicin
Colon cancer						
	−	63.1 ± 25.3	63.4 ± 26.3	74.7 ± 9.6	73.0 ± 19.9	70.7 ± 12.2
	+		69.5 ± 21.5	77.3 ± 11.6	77.3 ± 18.3	77.4 ± 8.3
Rectal cancer						
	−	53.8 ± 37.4	74.2 ± 14.9	73.2 ± 16.0	78.5 ± 13.2	56.5 ± 26.2
	+		85.9 ± 8.8[a]	75.8 ± 22.7	86.7 ± 11.5[a]	81.2 ± 10.9

*Percent inhibition was performed in combination with SN-38 and CDDP, FU, mitomycin, and doxorubicin. The values show an average ± SD.
[a]The inhibition rates of the combination with SN-38 plus CDDP and SN-38 plus MMC were significantly higher than that of these agents alone ($P < 0.01$).

there was no statistical significance of anticancer effectiveness when combining SN-38 and four other anticancer agents.

fp Concept in Combination With SN-38

The anticancer effect of SN-38 in combination with CDDP, FU, mitomycin, and doxorubicin was analyzed by means of the fp concept for freshly isolated colorectal cancer cells. As shown in Materials and Methods, fp > 1.0 means there is a synergistic antitumor effect. For rectal cancer, combining SN-38 and CDDP showed a 1.1 ± 0.2 fp value. However, other combinations for rectal and colonic cancer did not result in fp > 1.0 (Table III).

Lower Concentrations of SN-38 Augmented Antitumor Effects

For established colon cancer cell lines, LoVo and WiDr, the antitumor effects of SN-38 and CDDP in combination were analyzed using the fp concept. Low concentrations of SN-38 and CDDP showed a synergistic antitumor effect for LoVo (Table IV). A combination of 0.001 μg/ml SN-38 and 1 μg/ml CDDP indicated the most synergistic antitumor effect according to the fp concept. SN-38 at 0.1 μg/ml revealed a synergistic antitumor effect at various concentrations of CDDP for WiDr (Table IV).

Antitumor effects in combination with various concentrations of SN-38 and CDDP were analyzed in highly purified and freshly isolated colon cancer cells by way of the fp concept. Table V indicates that at lower than plasma peak concentrations of SN-38, the antitumor effect was augmented in combination with lower concentrations of CDDP. In case 1 in particular, combinations with 0.0001 and 0.001 μg/ml SN-38 and 0.01 and 0.1 μg/ml CDDP displayed the strongest synergistic antitumor effects.

TABLE III. Fractional Product Concept of SN-38 Combined with Other Anticancer Agents in Freshly Isolated Colorectal Cancer Cells*

	fp concept			
	CDDP	FU	Mitomycin	Doxorubicin
Colon cancer	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Rectal cancer	1.1 ± 0.2	0.8 ± 0.3	0.9 ± 0.1	0.9 ± 0.1

*The fp concept was referred to in Materials and Methods; fp >1 = synergistic, 1 = additive, <1 = antagonistic antitumor effect between two agents.

TABLE IV. Fractional Product Concept of Combination of SN-38 and CDDP in Colorectal Cancer Cell Lines*

		SN-38 (μg/ml)			
Cell line	CDDP (μg/ml)	0.0001	0.001	0.01	0.1
LoVo					
	0.01	NT	NT	1.1	1.0
	0.1	NT	NT	1.2	1.0
	1	2.0	3.5	1.3	1.1
	10	1.0	1.0	1.0	1.0
WiDr					
	0.01	NT	NT	NT	4.3
	0.1	NT	NT	NT	3.9
	1	NT	NT	NT	3.2
	10	NT	NT	NT	4.0

*NT = not tested. The fp concept was referred to in Materials and Methods; fp >1 = synergistic, 1 = additive, <1 = antagonistic antitumor effect between two agents.

DISCUSSION

FU–based chemotherapy is a standard regimen for colorectal cancer. However, cases of colorectal cancer with a FU failure require second-line chemotherapy. CPT-11 is a good candidate for second-line chemotherapy for colorectal cancer [1] because CPT-11 does not show a cross-resistance to CDDP and FU. As seen from clinical trials for advanced colorectal cancer, the response rate was 20.5%–32%, and the mean response periods were 5.6–10.6 months [26]. Moreover, it was not difficult to manage the main side effects, neutropenia and diarrhea, by using granulocyte colony-stimulating factor and loperamide hydrochloride optimally [26]. Thus, CPT-11 is considered to be an attractive anticancer agent for colorectal cancer [1], and it has already been established as a second-line chemotherapy for advanced colorectal cancer [27,28].

TABLE V. Fractional Product Concept of Combination of SN-38 and CDDP in Freshly Isolated Colorectal Cancer Cells*

		SN-38 (μg/ml)			
Case no.	CDDP (μg/ml)	0.0001	0.001	0.01	0.1
Case 1					
	0.01	20.8	12.9	6.6	1.0
	0.1	22.9	15.3	6.6	1.0
	1	24.9	29.6	6.3	0.9
	10	4.3	4.4	3.3	1.0
Case 2					
	0.1	NT	0.7	1.0	1.3
	1	NT	4.5	1.4	2.7

*NT = not tested. The fp concept was referred to in Materials and Methods; fp >1 = synergistic, 1 = additive, <1 = antagonistic antitumor effects between the two agents. Case 1 was a 76-year-old man with sigmoid colonic cancer; case 2 was a 53-year-old woman with sigmoid colonic cancer.

In order to promote the clinical response of CPT-11 in combatting colorectal cancer, one of the most effective strategies is a combination chemotherapy with other anticancer agents. In phase I and II clinical trials for advanced gastric cancer in Japan, CDDP was used in combination with CPT-11, and the response rate was >40% [29]. For advanced colorectal cancer, FU [30] and CDDP [13] were used in combination with CPT-11.

However, there was not any reasonable or theoretical way to choose the anticancer agents in combination with CPT-11, and established tumor cell lines were used for the analysis of the combination chemotherapy. In the present study, we examined the antitumor effectiveness of SN-38 in combination with other conventional anticancer agents by an MTT assay of highly purified, freshly isolated human colorectal cancer cells. We truly believe that one of the most important factors in the determination to choose anticancer agents is the use of freshly isolated and highly purified tumor cells [21,22]. An MTT assay was used to measure the anticancer effects and was found to be suitable for clinical samples because it was a rapid, easy, quantitative colorimetric system for determining chemosensitivity in vitro [18,19].

SN-38, in combination with CDDP and mitomycin, showed a high anticancer effect as compared with each anticancer agent alone as it related to freshly isolated rectal cancer. However, four conventional anticancer agents for colon cancer, and FU and doxorubicin for rectal cancer, did not augment the anticancer effect in combination with SN-38. We do not have a sufficient explanation for the different chemosensitivities between colon and rectal cancer.

To analyze further, the fp concept (fp value) was used to determine the synergy with CPT-11. The fp concept was not difficult, and furthermore it did not require many points to measure [24,25]; therefore, in terms of clinical samples, this method might be appropriate for the determination of effectively combined anticancer agents with CPT-11. By fp value, CDDP had a synergistic effect in combination with SN-38. However, mitomycin, FU, and doxorubicin did not show a synergistic effect in combination with SN-38. It is difficult to explain the discrepancy of mitomycin between the percent inhibition rate and the fp value. However, it was obvious that CDDP augmented the antitumor effect of SN-38 and that the augmentation was synergistic for freshly isolated colorectal cancer. These data may be supportive of ongoing clinical studies of chemotherapy by using CPT-11 and CDDP for advanced colorectal cancer. The mechanism of the synergy between CPT-11 and CDDP is not well analyzed. The resistant mechanism of both anticancer agents may be different, and both agents may induce apoptosis of tumors via different pathways [31].

To analyze the mechanism for augmentation between CPT-11 and CDDP, various concentrations of both anticancer agents were measured. At lower than plasma peak concentrations (>100 times lower concentrations than maximum concentration [C_{max}]) of SN-38, the anticancer effect of SN-38 was augmented in combination with lower concentrations (>10 times lower concentrations than C_{max}) of CDDP for freshly isolated colorectal cancer. Furthermore, this augmentation showed a strong synergistic effect. These results demonstrated two important matters for the chemotherapy of advanced colorectal cancer. The first is that the clinical efficacy of CPT-11 might be promoted in combination with CDDP. The second is that in combination with CDDP, there is the possibility of minimizing the side effects of CPT-11 by reducing the dose of CPT-11 without decreasing the antitumor effect for advanced colorectal cancer.

In the present study, we have demonstrated that CDDP is an attractive anticancer agent in combination with CPT-11 for advanced colorectal cancer and that in low-dose combinations with CPT-11 and CDDP, there exists a new strategy for fighting colorectal cancer without severe side effects. A further analysis, as it relates to timing and sequence, is needed to clarify the mechanism of the synergy between CPT-11 and CDDP.

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